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THE UNIVERSITY OF ALBERTA

Targeted transposition at the vestigial locus in *Drosophila melanogaster*

by

Tim Heslip

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILSOPHY

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL, 1994

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ISBN 0-315-95201-6

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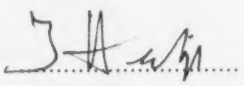

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June 27, 1994

This thesis is dedicated to my parents for their love and for the unending support they provide for the education of their children and others', and, to Nancy for making the hardest years of my life the best.

ABSTRACT

Targeted transposition is the replacement of one *P* element with another. This unique property of *P* elements often results in a change in phenotype of the target locus. Targeted transposition may be useful for circumventing the problem of position effects which act on transformed constructs. Position effect is the dependency of gene expression on the location of insertion. *P* element constructs targeted to the same site in the genome will be subjected to the same position effect, if any. This will allow subtle changes in gene expression to be ascribed directly to changes in regulatory sequence.

Some of the parameters affecting targeted transposition of a *Dopa decarboxylase*-containing transposon, *P*[*Ddc*], into a *P* element allele at the *vestigial* locus are examined herein. The donor element, *P*[*Ddc*], was targeted into vg^{21}, a cryptic *P* element-induced mutant allele of the *vestigial* (*vg*) locus. These events were detected by an increased mutant *vg* phenotype. The first targeted allele, vg^{28w}, contained the expected *P*[*Ddc*] plus an additional 9.5 kb of DNA, captured from elsewhere on chromosome II. Reversion of the vg^{28w} mutant allele demonstrated that the entire insert can excise but cannot reinsert at an appreciable frequency. The formation of chimeric elements may be an important component of *P* element dependent genomic instability.

In experiments producing higher frequencies of targeted transposition, the multiply inverted balancer chromosome, *CyO*, was heterozygous to the target. The location of the donor transposon *in cis* or *in trans* to the target had little effect on the frequency of targeting. Likewise, the mobility of different donor elements, as measured by their rate of transposition to a different chromosome, varied nearly 20-fold while the rate of targeted transposition was very similar between them. The *Ddc* gene in two of the three targeted alleles examined was internally deleted.

The presence of a non-inverted chromosome containing a vg^{+} allele heterozygous to the target results in the highest rate of targeted transposition. In this experiment, 26

targeted alleles were recovered from 2335 chromosomes scored. The presence of a non-inverted chromosome containing a deficiency for *vg*, heterozygous to the target resulted in very low targeted transposition giving 1 targeted allele from 2625 chromosomes scored. Most targeted alleles were precise replacements of the target *P* element by *P*[*Ddc*], but in several cases the donor was inserted in the opposite orientation and several alleles were missing one end of the donor transposon. Two targeted alleles which contained complete *P*[*Ddc*] were assayed for DDC activity and found to contain near normal levels of the enzyme. The results are discussed in light of a general model for targeted transposition. The targeted alleles could be described as the result of a replicative, conversion-like event.

Acknowledgements

I wish to thank those who helped out during the course of my graduate training. First, Ross, who took a chance, handed me the ball and trusted me to run with it. The three Bills (Clark, Brook and Addison) for their technical, intellectual, and moral support and for their friendship. To all my lab-mates, present and past, for putting up with my eclectic taste in music and sometimes forceful views on life, 'D', Adam and Xu Hong, Alberto, Gwenyth, Mark, Millan, Michelle, Jana and Andrew. The undergraduate students who suffered through countless single fly extractions, Dena, Mark, and Chris. To Nancy for helping on and off the field. To Sandra and Don for excellent DDC assays. To those who wrote letters of reference, Dave P., Dave N., John, Mike and Ross. To my fellow graduate students, past and present, for their friendship, thoughts and collaborations, Brian, Andrew, Audrey, Sita, Ali, and all the rest. Last but not least, to my mentor of molecular genetics, the one with the original observation on vg^{21}, for teaching me some science, occaisionally letting me win a pool game and showing me fine porcine appreciation, Jim.

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List of Symbols, Nomenclature or Abbreviations

bp	basepair
kb	kilobasepair
Mb	megabasepair
Ci	Curie
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
DNA	deoxyribonucleic acid
hr	hour
kb	kilobase
mg	milligram
ml	milliliter
mmol	millimole
ng	nanogram
nm	nanomole
SDS	sodium dodecylsulfate

µl	microliter
µg	microgram
λ	lambda

INTRODUCTION

The *P* elements of *Drosophila melanogaster* are one of the most studied and utilized eukaryotic transposable elements (for a review see Engels 1989). The first indication of the existence of this family of transposable elements was unexpected cross-overs in the male progeny of certain interstrain hybrids (Hiraizumi 1971). Cross-overs are normally restricted to females in *D. melanogaster*. This feature and several other unusual properties of these interstrain hybrids formed a syndrome which became known as hybrid dysgenesis (Kidwell *et al.* 1977). Once *P* elements were cloned, a molecular basis for hybrid dysgenesis was established (Bingham *et al.* 1982; Rubin *et al.* 1982). More important to the *D. melanogaster* scientific community as a whole, however, was the revolution in the way research was conducted in this organism. What follows is a brief introduction into the background of *P* elements from which the purpose of this thesis, a general study on targeted transposition at the *vestigial* locus, becomes clear.

The history of *P* elements began in the early 1970's when Hiraizumi and co-workers established the existence of chromosomes in wild populations that conferred meiotic recombination in hybrid males (Hiraizumi 1971; Hiraizumi *et al.* 1973). These males were the progeny of crosses between laboratory stock females and certain wild male parents. The chromosomes were called MR elements and were later shown to induce mutations as well (Golubovsky *et al.* 1977; Green and Shepherd 1979). The collection of phenotypes observed in the progeny of interstrain crosses was termed hybrid dysgenesis (Kidwell *et al.* 1977). The properties of this syndrome include increased mutability, male cross-overs, chromosomal aberrations, and temperature sensitive gonadal sterility. It was also noted that hybrid dysgenesis occurred only in crosses of males from wild populations to females of laboratory stocks and not the reciprocal. The wild strains were called P strains for their paternal activity and the laboratory strains were called M

strains for their maternal activity. The term cytotype was coined to explain the repression of hybrid dysgenesis in the oocytes produced by females of P strains (Engels 1979; Engels and Preston 1979, 1981). Hybrid dysgenesis is tightly regulated according to cytotype and is repressed in wild P strains. This repressed state explains the ability of *P* elements to spread throughout wild populations without killing their hosts.

The demonstration that hybrid dysgenesis was due to transposable elements began with the creation of mutants of the *white* (*w*) locus in a dysgenic cross (Rubin *et al.* 1982). The previously cloned *w* DNA was then used to clone the new mutant alleles which were shown to be the result of insertions of various sizes, named *P* elements. Immediately after cloning *P* elements, the same group of researchers demonstrated that hybrid dysgenesis is associated with multiple transpositions and suggested that the phenotypes of hybrid dysgenesis were caused directly or indirectly by *P* element activity (Bingham *et al.* 1982). With the *P* element DNA available, a wave of reports appeared which utilized hybrid dysgenesis to clone genes of interest (Searles *et al.* 1982; Williams and Bell 1988; for a review see Engels 1989). The molecular revolution in *D. melanogaster* had begun.

The first manipulation of *P* elements allowed for the development of a technique useful in any organism, namely germline transformation. The largest cloned *P* element, 2.9 kb in size, was able to transform a strain devoid of any *P* elements after injection into pre-blastoderm embryos (Spradling and Rubin 1982). These results and other experiments (Rubin and Spradling 1982) demonstrated that the 2.9 kb element was necessary and sufficient for transformation and that this element causes transposition of itself and smaller *P* elements. The sequence of this large autonomous *P* element revealed 31 bp inverted terminal repeats and four long open reading frames (O'Hare and Rubin 1983). Subsequent mutagenesis experiments demonstrated that the 31 bp inverted terminal repeats, and several hundred bases internal to the terminal repeats were necessary

for efficient transposition (Karess and Rubin 1984; Mullins *et al.* 1989). The sequences internal to the termini could not be exchanged and therefore are functionally different (Mullins *et al.* 1989). The fact that the 2.9 kb element contained the coding sequences for a trans-acting transposase was established genetically (Engels 1984) and it was later shown that a germline-specific splice between the last two exons of this element was responsible for the production of the transposase protein (Laski *et al.* 1986). Without this germline specific splice, a putative repressor protein is produced which suppresses hybrid dysgenesis. Related repressors are also produced from smaller internally deleted *P* elements which are abundant in P strains (Rio 1991). A small internally deleted *P* element was eventually used to make all transformation vectors, many of which carry the original ry^+ gene or a copy of the w^+ gene as a transformation marker (O'Hare and Rubin 1983). The immediate influence of transformation was the ability to study the regulation of gene expression *in vivo*.

A wide variety of techniques has been developed using *P* elements. For example, *P* elements have been used to create deletions of genomic sequences next to an insertion site (Voelker *et al.* 1984; Tsubota *et al.* 1985) and sequences of genes within a transformed *P* element (Daniels *et al.* 1985) in order to determine important regulatory regions. Indeed, the first report of a hybrid dysgenesis-induced deletion appeared before *P* elements were actually cloned. It was large enough to be cytologically visible (Green and Shepherd 1979). Large deletions of several Mb were later created between two adjacent *P* elements containing different marker genes inserted at specific cytological locations on the same chromosome (Cooley *et al.* 1990). Another technique has made use of a *P* element containing a bacterial origin of replication, an antibiotic resistance marker, and a β-galactosidase reporter gene. This construct, called an enhancer trap (O'Kane and Gehring 1987), has become very popular for its ability to detect regulatory elements which confer spatially and/or temporally distinct patterns of expression. Once an

insertion with an interesting pattern of β-galactosidase expression is found, the surrounding genomic sequences can be cloned by plasmid rescue. The techniques mentioned here are only a sample of those available.

After the discovery of the germline specific splice between the last two exons of the 2.9 kb autonomous element, constructs were made lacking the last intron (Laski *et al.* 1986). When transformed, these constructs produced transposase constitutively in both the soma and germline. Previously, *P* element activity was restricted to the germline because of the tissue specific splice. These constructs were called *P*[ry^+ Δ2-3], which signifies the deletion of the third intron and the presence of the marker gene, ry^+. One of the transformants recovered contained an immobile copy of this construct inserted at the cytological position 99B (Robertson *et al.* 1988). This created a constant source of transposase that could be introduced by mating and then removed in one generation. By using the *P*[ry^+ Δ2-3](99B) construct (hereafter referred to as Δ2-3) and strains containing only *P* elements of choice, transposition can be regulated even more tightly than when using typical P and M strains.

One of the problems associated with the use of *P* element transformation was that transgenes were subject to a previously known phenomenon of *D. melanogaster* called position effect. Position effects were noticed with the first gene, *rosy* (*ry*), that was re-introduced into the genome (Rubin and Spradling 1982). In short, position effect is the variation in expression due to genomic location. While some transgenes appeared at first to be relatively insensitive to position effects, almost all transformed contructs can be affected in at least subtle ways (Scholnick *et al.* 1983; Chen and Hodgetts 1987). Transformation allows for studying the regulation of gene expression at a gross level by engineering changes into potential regulatory sequences and then analyzing expression after re-introduction into the genome. However, subtle contributions to the control of

transcription are difficult or impossible to separate from position effects. It is this fact which prompted the research undertaken in this thesis, as outlined below.

P element mediated transformation has been particularly difficult to use for studying the regulation of expression of the *Dopa decarboxyalse* (*Ddc*) gene in the epidermis of *D. melanogaster*. The epidermal expression profile of *Ddc* is complex and follows or coincides with the peaks of ecdysone titers that occur during development. While hormonal regulation of *Ddc* expression at pupariation may be mediated through protein products of the *Broad* complex which bind to the first intron of *Ddc in vitro*, there is also a set of redundant *cis*-acting sequences upstream of the *Ddc* promoter which are at least in part responsible for the regulation of the gene (Scholnick *et al.* 1986). The contribution of each of these sequences is similar in magnitude to the variation in expression due to position effects. Also, transformation of a wild type *Ddc* allele has resulted in insertions which vary in their expression of *Ddc* several fold (Chen and Hodgetts 1987). In order to study the role of the subtle contributions of such regulatory sequences, either position effects must eliminated or a control must be found for them.

One way to eliminate position effects is to change the sequences of interest at the endogenous locus. The only way to do this in *D. melanogaster* is by targeted gene replacement (Gloor *et al.* 1991). While studying reversion of a *P* element induced allele of the X-linked *w* locus, w^{hd}, using Δ2-3, Engels and co-workers discovered that the frequency of reversion was dependent on the type of homologous chromosome that was heterozygous to w^{hd} (Engels *et al.* 1990). In their experiments, reversion of w^{hd} required either a precise reversion of the insertion restoring the wild type w^{+} sequence. If the reversion was performed in a male or female hemizygous for the site of insertion, the frequency was significantly less than when performed in a female heterozygous for wild type sequences. In addition, reversion in males was more frequent when an ectopic copy

of w^+ was present on the same chromosome. The data suggested to the authors that after the *P* element of w^{hd} excised, a double strand gap repair mechanism was induced to fill in the site and that an ectopic copy of the gene could be used as a template for the repair.

Acting on the reversion experiments at w^+ (Engels *et al.* 1990), Gloor *et al.* (1991) used an ectopic copy of the w^+ gene, which contained silent substitutions in exonic sequences, to demonstrate that when w^{hd} is reverted, polymorphisms can be converted into the w^+ locus by targeted gene replacement. This fact supports the concept of a template based repair mechanism acting after excision of a *P* element. The larger implication of this experiment and those to follow was that sequences could be altered at the endogenous locus without many of the problems previously associated with transforming large constructs.

Targeted gene replacement requires that a *P* element insertion exists near the sequence to be altered and that the *P* element allele has a detectable phenotype that changes when the insertion is lost. Neither a *P* element allele nor a readily observable phenotype is available for *Ddc*. Therefore, to control for position effects in studies of the regulation of *Ddc* expression, a method other than targeted gene replacement was sought. The new method was named targeted transposition and the purpose of this thesis was to study the variables that effect this newly described property of *P* elements.

The phenomenon of targeted transposition was first noted at the *vg* locus (Williams *et al.* 1988). The *vg* locus was tagged with a small (687 bp) *P* element using hybrid dysgenesis (Williams and Bell 1988). This mutant allele, vg^{21}, was very weak, or cryptic. In order to obtain stronger alleles and possibly uncover important promoter or regulatory sequences near *vg*, the vg^{21} allele was subjected to further hybrid dysgenesis in the hope of creating deletions near the insertion site as had been previously reported for

other loci (Voelker *et al.* 1984; Tsubota *et al.* 1985). Surprisingly, some of the new stronger alleles contained larger insertions at the exact position as the original vg^{21} *P* element (Williams *et al.* 1988). This unusual property then raised the possibility of using the increase in mutant *vg* phenotype to detect the recruitment of larger *P* elements containing genes of interest to the *vg* locus. Then, all constructs of any given gene would be subjected to the same position effect, if any, and the changes made in the genes could be ascribed directly to changes in gene expression. This type of gene targeting has been termed targeted transposition for two reasons (Heslip *et al.* 1992); the phenomenon resembles gene conversion and the only sequence homology between the target and the donor element is *P* element DNA.

The use of gene targeting in mammals and *D. melanogaster*, whether by targeted gene replacement or targeted transposition, is a study of contrasts. In the first case, gene targeting is used more often to create 'knock out' mutations in mice in order to study a gene that has been cloned but for which the function can only be inferred (Joyner *et al.* 1989). The regulation of a cloned gene is more conveniently studied in cell culture in mammals. In the case of *D. melanogaster*, mutations are more readily acquired and thus are created first, usually in screens for functions which cause a specific phenotype when they are defective. Then, the gene is usually cloned and its regulation studied using *P* element mediated transformation, in which constructs are analyzed in the organism itself. In *D. melanogaster*, gene targeting has only recently become available, and more recently feasible, as a technique for studying the regulation of gene expression (Nassif *et al.* 1994).

In summary, targeted transposition is the replacement of one *P* element with another. The replacement is precise, leaving the local genomic DNA unchanged. This event can result in an increase in mutant phenotype as reported at the *P* element-induced hypomorphic alleles of the *vestigial* and *yellow* loci, vg^{21} and $y^{13\text{-}11}$ (Geyer *et al.* 1988;

Williams *et al.* 1988). The increase in mutant phenotype is the basis for detecting targeted events.

This thesis consists of four main parts. The first Chapter details the production and molecular characterization of the first targeted transposition. This new allele of *vg* was the result of a 19 kb chimeric insertion, half of which was the donor transposon that was intended to be targeted to *vg*. This unusual insertion at *vg* was analyzed in terms of its ability to behave like a true transposable element and its relationship to the evolution of transposable elements and the genome. The second Chapter is an investigation of variables which affect targeted transposition including the location of the donor element to be targeted and the ability of these donors to mobilize. Some sequence characterization of the nine targeted alleles is also presented in light of potential mechanisms for targeted transposition. The third Chapter studies the effect of different second chromosome homologues on the rate of targeted transposition. The ability of the vg^{21} *P* element to transpose under these different conditions is also included in relation to rates of targeted transposition. This Chapter also includes an analysis of the expression of the first targeted *Ddc* transposon at the level of enzyme activity in order to determine the feasibility of using *vg* as a target and circumventing the problems of position effects. Finally, the general discussion will include a model to explain the mechanism of targeted transposition which is based on data presented here and by others.

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Chapter 2

Targeted Transposition from Different Donor Locations

INTRODUCTION

Using the enhanced mutant phenotype of *vg* as the basis of a targeting scheme, a *P* element transposon carrying the *Ddc* gene, *P*[*Ddc*], and one carrying an enhancer trap have been recovered at *vg* (Heslip *et al.* 1992; Staveley *et al.* 1994). The work presented in this Chapter was initiated to assess various parameters that might influence the rate of targeted transposition. The effect of the location of the donor transposon in a *cis* or *trans* configuration relative to the target, vg^{21}, was examined. The transposition rate of different donor transposons was measured to determine if there is a relationship between the mobility of a donor transposon and the rate of targeting. The analyses indicate that all targeted alleles were precise replacements of the resident vg^{21} *P* element by the donor. Some targeted alleles contained the donor *P* element in the opposite orientation relative to the original vg^{21} *P* element. Two of the three alleles examined by Southern hybridizations contained an internally deleted *P*[*Ddc*]. The location of the donor element, *in cis*, *in trans*, or on a non-homologous chromosome, and its transposition frequency had little influence on the rate of targeted transposition.

MATERIALS AND METHODS

***Drosophila melanogaster* stocks :** All crosses were performed on yeast medium at 22° to 24° unless otherwise indicated (Nash and Bell 1968) and in batches unless otherwise noted. Ddc^{ts2} and Ddc^{n7} are temperature sensitive and recessive null alleles, respectively, of the *Ddc* gene (Wright *et al.* 1981). The vg^{21} allele is a *P* element insertion into the *vg* locus and vg^{79d5} is caused by a small deletion within the locus (Williams and Bell 1988). The third chromosomes, *TM2, Ubx ry P*[ry^{+} Δ2-3](99B) and *Sb* ry^{506} *P*[ry^{+} Δ2-3](99B), contain a stable genomic source of transposase (Robertson *et*

al. 1988) and will be referred to as *TM2*[Δ2-3] and *Sb*[Δ2-3] respectively. The donor, *P*[*Ddc*], is a *P* element vector, pHDlac, containing a 7.6-kb *Pst*I fragment that includes the *Ddc* gene (Chen and Hodgetts 1987). The *P* element donor carried on the X chromosome, *P*[inv*Ddc*], contains the same 7.6-kb *Pst*I *Ddc* fragment as *P*[*Ddc*] but in the opposite orientation relative to the *P* element vector sequences (Marsh *et al.* 1985). Other mutant alleles and chromosomes are described in (Lindsley and Zimm 1992).

DNA manipulations: Culturing and storage of bacteria, preparation of plasmid DNA, and restriction digestions were performed according to standard methodology (Maniatis *et al.* 1982). *D. melanogaster* genomic DNA for Southern hybridization was isolated as in Heslip et al.(1992).

Genomic DNA to be used for DNA amplification was isolated as follows: Flies were homogenized in 50 µl of 10 mM Tris-Cl pH 7.5, 60 mM EDTA, 0.15 mM spermidine, 0.15 mM spermine, and 100 µg/ml proteinase K. After incubating the homogenate for 30 to 60 min. at 37°, 50 µl of 0.2 M Tris-Cl pH 9.0, 30 mM EDTA, 2% SDS was added and incubated at 65° for 30 min. with occasional agitation. A single organic extraction was performed; 150 µl of 1:1 phenol/chloroform was added, mixed and warmed to 65° with occasional agitation. After brief centrifugation, 80 µl of the aqueous phase was subjected to DNA purification using GeneClean (Bio 101 Inc.) according to the manufacturer's instructions. The purified DNA was resuspended in 30 µl of TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Genomic DNA was extracted from single flies that were used to establish lines (see RESULTS). Flies with a small increase in mutant *vg* phenotype were pooled using 2-4 individuals in a single DNA extraction.

Southern hybridizations: Restriction digested DNA was fractionated by agarose gel electrophoresis and transferred onto Genescreen Plus membranes (Dupont). Hybridizations were performed at 42° in 50% formamide according to the manufacturer's instructions using oligolabeled DNA restriction fragments (Feinberg and Vogelstein 1983) purified from agarose gels with GeneClean (Bio 101 Inc.).

DNA sequencing: DNA amplification products were purified from agarose gels with GeneClean and sequenced using the ABI Taq DyeDeoxy Terminator Cycle Sequencing Kit according to the manufacturer's instructions. The sequencing reactions were fractionated and analyzed on an ABI 370A DNA sequencing apparatus.

DNA amplification: Each amplification reaction used 3 µl (approximately 50 ng) of DNA in a total volume of 30 µl which contained 2-3 U of Taq polymerase (Bio-Can or BRL) and a final concentration of 50 mM Tris-Cl (pH 9.2), 1.5 mM $MgCl_2$, 0.005% ß-mercaptoethanol, 0.1 µg/µl bovine serum albumin (Boehringer Mannheim), 200 µM of dATP, dCTP, dGTP, dTTP, and 0.3 ng/µl of primer. Reactions were run in a Stratagene Robocycler 40. The amplification program started with 5 min at 95°, 1.5 min at 60°, and 3 min at 73°, followed by 29 cycles of 1 min at 93°, 1 min at 60°, and 2-3 min at 73°. All amplification products were run on agarose minigels at 70 V. Primers used are listed by numbers as they appear in the results: #1; 5'-ATCCCGCGCGGCGGTGAGAG-3', #2; 5'-TTCGGAGCGTGATGTTGACA-3', #3; 5'-ATCGGCGTTGTAAAGACTGC-3', #4; 5'-GTACTCCCACTGGTATAGCC-3', #5; 5'-CGTCGAAAGCCGAAGCTT-3', #6; 5'-AATCAAGTGGGCGGTGCTTG-3'.

RESULTS

Targeting in a *cis* configuration: In the previous Chapter, the donor and target were on homologous chromosomes in a *trans* configuration (Heslip *et al.* 1992). We obtained one targeted *vg* allele, vg^{28w}, out of 18,500 flies scored. This low frequency led us to examine whether targeted transposition would occur faster if the donor and the target were in a *cis* configuration. To test this, the crosses shown in Figure 1 were set up to target the *P*[*Ddc*] at 21B into vg^{21} at 49D on the same chromosome. The germline of the males in the second cross contains the target (vg^{21}), the donor transposon (*P*[*Ddc*]), and the transposase source ([Δ2-3]). Some of the progeny from these males could contain

Figure 1:

Crossing scheme for targeting *in cis*. Males and females are indicated. The targeting crosses are designed to target *P*[*Ddc*], located at 21B, into vg^{21}. This occurs in the germline of the males in the second cross. The progeny from the second cross inheriting the putative targeted allele, vg^{a*}, are detected by their more extreme mutant *vg* phenotype and used to establish stocks. The *CyO* second chromosome is marked with *cn* and *pr*. The *TM2* third chromosome is marked with *Ubx* and *e*.

♀ $P[Ddc](21B)\ Ddc^{ts2}\ pr\ vg^{21}$ X ♂ $\frac{Ddc^{n7}\ pr}{CyO}$; $\frac{Sb[\Delta 2\text{-}3]}{TM2[\Delta 2\text{-}3]}$

♂ $\frac{P[Ddc](21B)\ Ddc^{ts2}\ pr\ vg^{21}}{CyO}$; $\frac{Sb[\Delta 2\text{-}3]}{+}$ X ♀ $\frac{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}{CyO}$

$\frac{P[Ddc](21B)\ Ddc^{ts2}\ pr\ vg^{a*}}{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}$; $\frac{Sb[\Delta 2\text{-}3]\ \text{or}\ +}{+}$

a targeted *vg* allele, denoted as vg^{a*} in Figure 1. The increase in mutant *vg* phenotype, indicative of a targeted event, is easily scored in the vg^{79d5} heterozygote, since vg^{21}/vg^{79d5} exhibits a weak phenotype. Any fly with a stronger mutant phenotype than vg^{21}/vg^{79d5} was mated to Ddc^{ts2} *pr cn* vg^{79d5}/*CyO* flies to establish a line over the *CyO* balancer chromosome based on the cn^{+} flies from this cross. Once a viable culture of larvae was established, the founding adult was frozen and used for single fly DNA amplification. These crosses and subsequent analyses constitute experiment 'a'.

Molecular maps of vg^{+} and vg^{21} are shown in Figures 2a and 2b. The cryptic vg^{21} allele is caused by the insertion of a 687 bp *P* element just upstream of the putative transcription start site (Williams *et al.* 1991). Figure 2c illustrates the molecular map of a typical targeted allele, vg^{a207}. The three primers used for single fly DNA amplification are also shown. These oligonucleotides are complementary to downstream *vg* (#1), 5' *Ddc* (#2), and 3' *Ddc* (#3) sequences respectively. Following the amplification reaction, a product will be observed only in flies bearing a targeted transposition. Using both *Ddc* primers and the *vg* primer ensures that a product will be seen regardless of the orientation of the *P*[*Ddc*] in a targeted allele. A total of 2980 chromosomes was scored from experiment 'a'. Genomic DNA from 221 flies with an increased mutant *vg* phenotype plus approximately 150 flies with borderline phenotypes was subjected to amplification. Table 1 shows that three targeted alleles, vg^{a43}, vg^{a202}, and vg^{a207}, were recovered. This rate of targeted transposition was roughly six fold higher than that found in our previous experiment using the same *P*[*Ddc*](21B) donor.

Targeting *in cis* using a more proximal donor: In the targeted gene replacement experiments of Gloor and co-workers (Gloor *et al.* 1991), the highest rate of conversion at the *white* locus was obtained using the closest donor template. To test the effect of a closer donor on targeted transposition, the crosses shown in Figure 3 were carried out in which the donor *P*[*Ddc*] was located at 35C. The progeny from the second cross were

Figure 2:

Restriction maps of the *vestigial* alleles in this Chapter. Restriction sites are as follows: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; T, *Sst*I. All *Eco*RI sites are shown but only informative sites are shown for the rest, including the characteristic *Hin*dIII at the 5' end of *P* elements. (a) The vg^{+} allele. The vg^{+} probe referred to in the text is the 0.7 kb *Eco*RI fragment from this gene. The wavy arrow indicates the 5' end of the first exon of *vg* (Williams *et al.* 1990). (b) The vg^{21} allele. The 5' and 3' ends of the *P* element are indicated below the solid arrows (◄——►) on the map. The 3' end of the *P* element is about 20 bp upstream of the first exon of *vg* (Williams *et al.* 1991). (c) The vg^{a207} allele. The 5' and 3' ends of the *P* element vector of *P*[*Ddc*] are shown above the map. The wavy arrow indicates the transcription start site of *Ddc* and therefore the polarity of the *Ddc* gene is opposite to that of the *P* element. The putative *vg* transcription start site is not shown but remains unchanged from (b). Restriction map symbols: *vg* genomic DNA, ——; *P* element DNA with a terminus,——►; *Ddc* exons, introns, and untranscribed genomic DNA, —▬▬—. The internal deletion of the *Ddc* gene in vg^{a207} is arbitrarily indicated by the round brackets, ( ). Below the restriction map are the locations and polarity (5' to 3') of the amplification primers whose sequences are listed in the MATERIALS AND METHODS. Below the primers are the locations of probes for Southern hybridizations. (d) The *P*[inv*Ddc*] donor. The orientation of the *P* element vector is the same as in (c); the 5' and 3' ends are shown above the map. The *Ddc* gene is cloned into this construct in the opposite direction compared to the *P*[*Ddc*] shown in (c). The restriction map symbols are the same as in (c). The location of amplification primers is shown below the map.

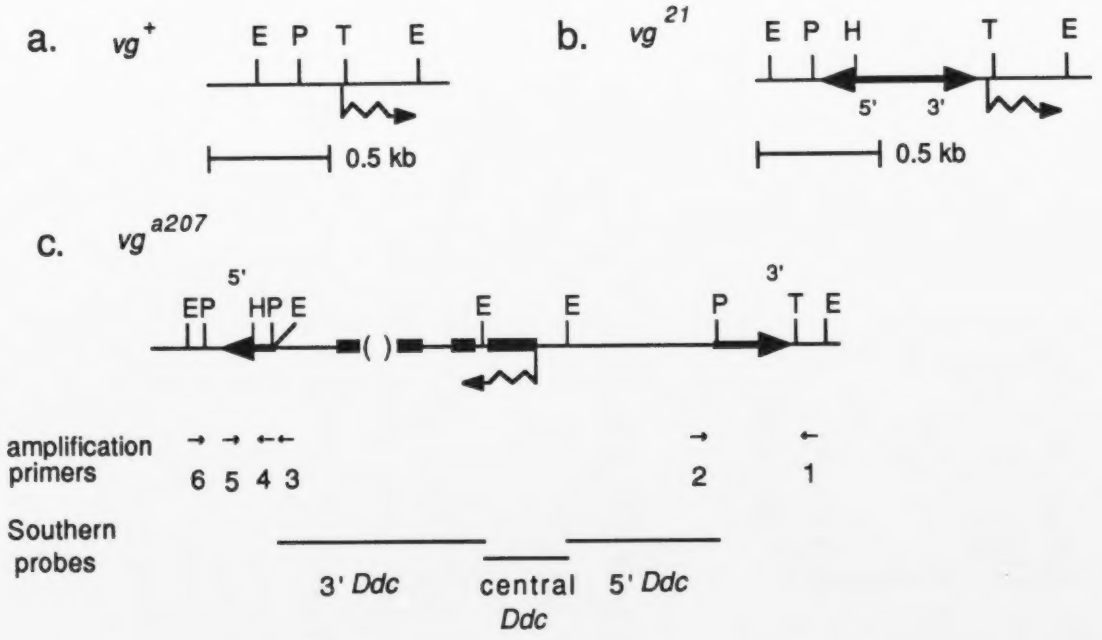

a.
vg+
E P T E
0.5 kb
b.
vg21
E P H T E
5' 3'
0.5 kb
c.
vg a207
5'
EP HP E
( )
E E
P
3'
T E
amplification primers
6 5 4 3
2
1
Southern probes
3' Ddc
central Ddc
5' Ddc
d.
P [inv Ddc]
5'
HP
E E
P
3'
5 4 2
3
1 kb

TABLE 1

DNA amplifications

Experiment	Line	Amplification reaction primers[a]			Chromosomes scored
		#1, #2, and #3	#1 and #4	#4 and #6	
a	a43	+	-	+	2980
	a202	+	-	+	
	a207	+	-	+	
f	f38	+	-	+	2258
	f49	+	+	-	
	fs1	+	-	+	
x	x335	+	-	+	1855
	x336	+	+	-	
	x347[b]	+	+	-	

[a] The reactions including primers #1, #2, and #3 were used in the initial screen for targeted alleles while the next two reactions using primers #1 and #4, and primers #4 and #6 were used to determine the orientation of donor in targeted alleles. A '+' indicates the presence of a DNA amplification product and a '-' the absence of a product.

[b] The founding parent of line x347 was used for these amplification reactions. When sublines x347.2 and x347.11, which were derived from x347 (see **Southern analysis**), were analyzed, the same results were obtained (data not shown).

Figure 3:

Crossing scheme for targeting *in cis* using a more proximal donor. The crosses are designed to target *P*[*Ddc*], located at 35C, into vg^{21}. The progeny from the second cross, which inherit a putative targeted allele, are denoted as vg^{f*}.

♀ $\frac{P[Ddc](35C)\ Ddc^{ts2}\ pr\ vg^{21}}{}$ X ♂ $\frac{Ddc^{n7}\ pr}{CyO}$; $\frac{Sb[\Delta 2\text{-}3]}{TM2[\Delta 2\text{-}3]}$

♂ $\frac{P[Ddc](35C)\ Ddc^{ts2}\ pr\ vg^{21}}{CyO}$; $\frac{Sb[\Delta 2\text{-}3]}{+}$ X ♀ $\frac{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}{CyO}$

$\frac{P[Ddc](35C)\ Ddc^{ts2}\ pr\ vg^{f*}}{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}$; $\frac{Sb[\Delta 2\text{-}3]\ \text{or}\ +}{+}$

scored for an increase in mutant *vg* phenotype and targeted alleles were confirmed using the same procedure as in experiment 'a'. The data are grouped as experiment 'f' in Table 1. Three targeted alleles, vg^{f38}, vg^{f49}, and vg^{fs1} were detected amongst the 320 flies that we analyzed with a borderline to clearly mutant phenotype after scoring 2258 chromosomes. This rate is comparable to experiment 'a' and suggests donor proximity has little influence on the rate of targeted transposition.

Targeting *in trans* from the X chromosome: As mentioned above, in the previous Chapter the donor *P*[*Ddc*] was located on the homologous second chromosome, *in trans* relative to vg^{21}, and the rate of targeted transposition was very low (Heslip *et al.* 1992). To test the generality of this result when using donors *in trans*, an experiment was set up using a donor on the X chromosome. The *Ddc* gene contained in the X-linked donor (Figure 2d) was cloned into the *P* element vector in the opposite orientation compared to *P*[*Ddc*] (Marsh *et al.* 1985). For this reason, the X-linked donor will be referred to as *P*[inv*Ddc*]. In experiments 'a' and 'f', the targeting was performed over the multiply inverted *CyO* chromosome and in order to be consistent, the targeting from the X chromosome was also carried out in a vg^{21}/CyO heterozygote. The crosses shown in Figure 4a were used to target *P*[inv*Ddc*] from 10C on the X chromosome into vg^{21} and the data are grouped as experiment 'x' in Table 1. Genomic DNA samples from 150 flies with an increase in mutant *vg* phenotype plus an additional 100 flies with a borderline phenotype were tested. Table 1 shows that three targeted alleles were generated in experiment 'x' from 1855 chromosomes scored. Thus, this *trans* configuration of this donor yielded a rate of targeting similar to those observed from *cis* donor locations.

Targeting *in trans* from the homologous second chromosome: DNA amplification was not used in the screening process of the targeting experiment in the previous Chapter. Using DNA amplification, faster screening and consequently the

Figure 4:

Crossing schemes for targeting *in trans*. (a) These crosses are designed to target *P*[inv*Ddc*], located at 10C on the X chromosome, into vg^{21}. The progeny from the second cross which inherit vg^{x*}, the putative targeted allele, are detected by their more extreme mutant *vg* phenotype and used to establish stocks. The *P*[inv*Ddc*](10C) containing X chromosome is marked with *y*. (b) The last cross from Figure 2a of Heslip *et al.* (1992) is shown. The crosses are designed to target *P*[*Ddc*](21B) from a *trans* position on the non-inverted homologous second chromosome into vg^{21}. This occurs in the germline of the male parents shown. The progeny inheriting a potentially targeted allele, vg^{t*}, are used to establish stocks.

a.

$$\text{♀}\ P[\text{inv}Ddc](10C)\ ;\ \frac{Ddc^{ts2}\ cn\ vg^{21}}{CyO}\ \text{X}\ \text{♂}\ \frac{Ddc^{n7}\ pr}{CyO}\ ;\ \frac{Sb[\Delta 2\text{-}3]}{TM2[\Delta 2\text{-}3]}$$

$$\text{♂}\ P[\text{inv}Ddc](10C)\ ;\ \frac{Ddc^{ts2}\ cn\ vg^{21}}{CyO}\ ;\ \frac{Sb[\Delta 2\text{-}3]}{+}\ \text{X}\ \text{♀}\ \frac{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}{CyO}$$

$$\frac{Ddc^{ts2}\ cn\ vg^{x*}}{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}\ ;\ \frac{Sb[\Delta 2\text{-}3]\ \text{or}\ +}{+}$$

b.

$$\text{♂}\ \frac{Ddc^{ts2}\ cn\ vg^{21}}{P[Ddc](21B)\ Ddc^{ts2}\ pr}\ ;\ \frac{Sb[\Delta 2\text{-}3]}{+}\ \text{X}\ \text{♀}\ \frac{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}{CyO}$$

$$\frac{Ddc^{ts2}\ cn\ vg^{t*}}{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}\ ;\ \frac{Sb[\Delta 2\text{-}3]\ \text{or}\ +}{+}$$

testing of more flies with a slight increase in mutant phenotype is possible. Therefore, the original *trans* experiment was repeated to determine whether or not targeted events might have been overlooked by selecting only strong *vg* adults. The same crosses were used as in Figure 3a of Chapter 1, the last of which is shown in Figure 4b. The results are listed in Table 1 as the 't' experiment. DNA from approximately 100 flies with an obvious increase in mutant *vg* phenotype plus another 100 flies with borderline phenotypes was tested. Out of 2209 chromosomes scored, no targeted transposition was detected.

The orientation and sequencing of targeted donors: The primary DNA amplification reaction used to detect targeted transposition, which included primers #1, #2, and #3 (Figure 2c), could discriminate between the possible orientations of a targeted donor based on the size of the amplification product. For example, when using *P*[*Ddc*] as the donor, if the *P* element portion of this transposon is inserted at *vg* in the same orientation as the original *P* element in vg^{21} then primers #1 and #2 will yield a 0.8-kb product. If *P*[*Ddc*] inserts in the opposite orientation then primers #1 and #3 will yield a 0.7-kb product. Similarly, when using *P*[inv*Ddc*], the relative orientation of a targeted donor can be determined based on the size of the product in the primary amplification reaction.

Products from the primary DNA amplification reactions suggested that the *vg* alleles *a43*, *a202*, *a207*, *f38*, *fs1*, and *x335* contained donor *P* elements in the same orientation as the original vg^{21} *P* element and that *vg* alleles *f49*, *x336*, and *x347* contained donor *P* elements in the opposite orientation to the original vg^{21} *P* element . To confirm the orientation of all targeted donor elements, primer #4 and either primer #1 (the downstream *vg* primer in Figure 2c) or primer #6 (the upstream *vg* primer shown in Figure 2c) were used in separate DNA amplifications of each targeted line. Primer #4 is complementary to *P* element sequences present in the donors but absent in the vg^{21} *P* element and directs synthesis toward the 5' *P* element terminus. In these reactions we

expect a product in only one of the two reactions carried out on each line. A product from primers #4 and #6 indicates that the targeted *P* element is in the same orientation as the original vg^{21} *P* element whereas a product from primers #4 and #1 indicates the targeted *P* element is in the opposite orientation. The results are shown in Table 1. The orientation of targeted donors in *vg* alleles *a43*, *a202*, *a207*, *f38*, *fs1*, and *x335* is the same as the original vg^{21} *P* element. The orientation of the targeted donors in *vg* alleles *f49*, *x336*, and *x347* is opposite to the original vg^{21} *P* element. The results of these DNA amplifications confirmed the predictions of the initial reaction on all targeted alleles.

The DNA amplification products from the reactions using primers #4 and #6 on vg^{a43}, vg^{a202}, vg^{a207}, vg^{f38}, vg^{fs1}, and vg^{x335}, were purified from agarose gels and sequenced. A sequence polymorphism that exists in *P* element vectors at nucleotide position 33 was used in Chapter 1 to demonstrate the replacement of the 5' end of the vg^{21} *P* element with the 5' end of *P*[*Ddc*] in vg^{28w} (Heslip *et al.* 1992). This polymorphism was again present in all these DNA amplification products, indicating that the 5' *P* element in these lines was derived from the donor elements and not the original *P* element of vg^{21}. The amplification products of vg^{f49}, vg^{x336}, and vg^{x347} using primers #1 and #4 were also sequenced. The polymorphism also present in the 5' *P* element sequences of these lines. This is expected if the 5' *P* element sequence of the donor has replaced the 3' *P* element sequence of vg^{21} because the sequence of the *P* element termini diverges immediately internal to the 31 bp inverted terminal repeats.

In addition to sequencing the DNA amplification products mentioned above, the opposite junction of each targeted insertion was also sequenced from appropriate DNA amplification products. In all cases, both junctions of the targeted insertions revealed precise replacement of the original vg^{21} *P* element with the new insertion leaving the 8-bp duplication flanking the insertion of vg^{21} and the rest of the *vg* sequence intact.

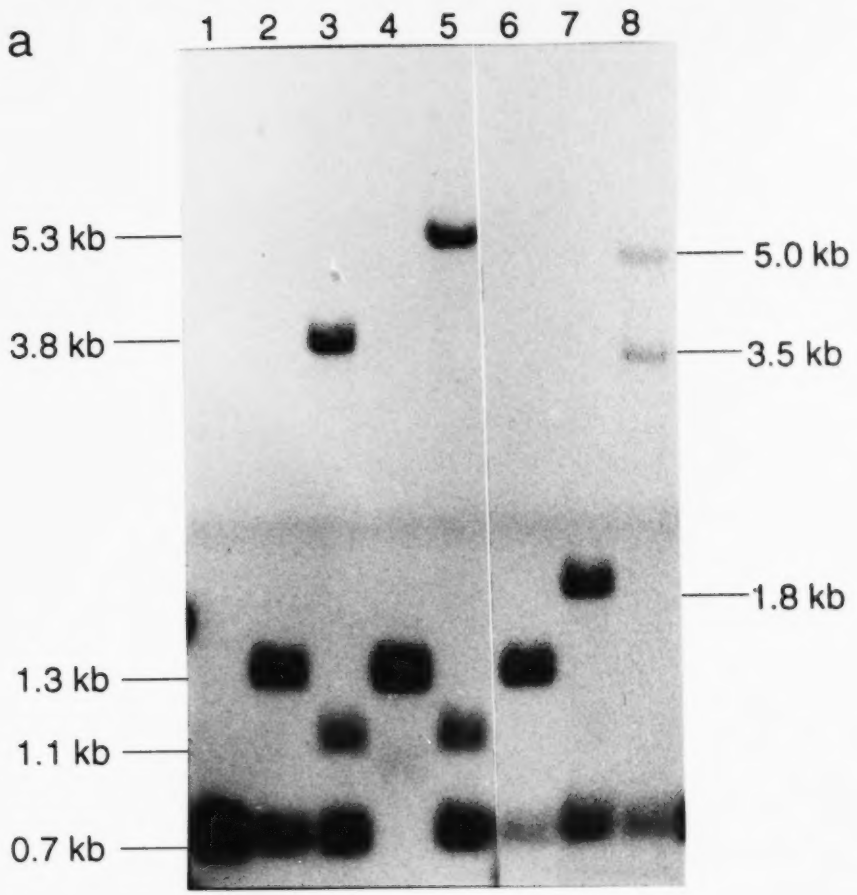
Southern analysis: The established lines, a207, f38, and x347, which contained targeted *vg* alleles, were analyzed by Southern hybridizations. In using DNA amplification to detect a targeted event, insertion is tested only for those *Ddc* sequences complementary to the *Ddc* primers at the *vg* locus. If a targeted event results in the deletion of either the *vg* primer sequence or the appropriate *Ddc* sequence then an amplification product would not be observed. For this reason, any lines that had a strong homozygous phenotype but did not yield an amplification product were also analyzed by Southerns and none of these proved to contain targeted alleles.

During the maintenance of line x347 and from an initial Southern blot (data not shown), it was apparent that more than one targeted *vg* allele was segregating from the stock. This is because the founding parent of line x347 inherited a *Sb*[Δ2-3]-containing third chromosome which probably caused the original targeted transposition to be followed by a rearrangement. To eliminate the heterogeneity in this stock, several males from line x347 that were heterozygous for *CyO* were used to make sublines. Based on a subsequent Southern and DNA amplifications, two sublines, x347.2 and x347.11, representing the two targeted *vg* alleles in line x347, were established and used for further analysis.

Lines a207, f38 , x347.2 and x347.11 were included on the Southern blot shown in Figure 5. Strains used as controls include those containing the donor and target from the first cross of each targeting experiment (Figures 2, 3, and 4a) and the wild type strain *Canton-S*. The genomic DNA samples were digested with *Eco*RI. The blot was hybridized with the 0.7-kb *Eco*RI fragment from vg^+ shown in Figure 2a (hereafter referred to as the vg^+ probe). The resulting autoradiograph is shown in Figure 5a. The 0.7-kb band seen in the first lane is due to the vg^+ allele present in the *Canton-S* control strain. The same band is present in the all other lanes (except lane 4, a strain that does not contain *CyO*) due to the vg^+ allele on the *CyO* chromosome. The control lanes 2, 4, and 6 show a 1.3-kb band due to the insertion of the 687-bp *P* element at *vg* as illustrated in

Figure 5:

Southern blot analysis. Genomic DNA was digested with *Eco*RI. The sizes of informative bands discussed in the text are indicated beside each autoradiograph. The lanes in (a) and (b) contain DNA from the following strains: Lane 1; *Canton-S*, lane 2; *P*[*Ddc*](21B) *pr* Ddc^{ts2} vg^{21}/*CyO*, lane3; *P*[*Ddc*](21B) *pr* Ddc^{ts2} vg^{a207}/*CyO*, lane 4; *P*[*Ddc*](35C) *pr* Ddc^{ts2} vg^{21}, lane 5; *P*[*Ddc*](35C) *pr* Ddc^{ts2} vg^{f38}/*CyO*, lane 6; *P*[inv*Ddc*](10C); Ddc^{ts2} *cn* vg^{21}/*CyO*, lane 7; Ddc^{ts2} *cn* $vg^{x347.2}$/*CyO*, lane 8; Ddc^{ts2} *cn* $vg^{x347.11}$/*CyO*. (a) Autoradiograph using the vg^{+} probe (Figure 2a). (b) The blot from (a) was stripped and re-hybridized with the 5' *Ddc* probe (Figure 2c). The double bands present in lanes 2,3, and 5 (discussed in **Southern analysis**) were confirmed using another Southern blot containing these strains in which the electrophoresis was run for a longer period of time (data not shown).


a
1 2 3 4 5 6 7 8
5.3 kb
3.8 kb
1.3 kb
1.1 kb
0.7 kb
5.0 kb
3.5 kb
1.8 kb

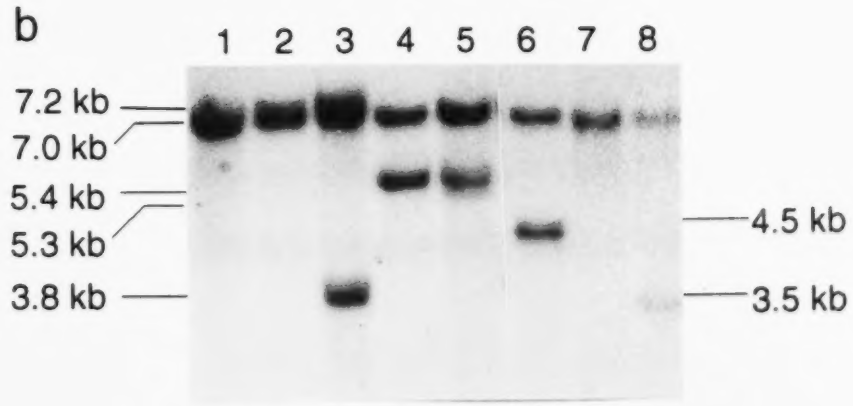

b
1 2 3 4 5 6 7 8
7.2 kb
7.0 kb
5.4 kb
5.3 kb
3.8 kb
4.5 kb
3.5 kb

Figure 2b. If a complete *P*[*Ddc*] is targeted to *vg* in either experiments 'a' or 'f', regardless of the orientation of the insert, a 1.1-kb band and a 3.5-kb band are expected on the autoradiograph. This is because the site of insertion at *vg* is centered between *Eco*RI sites (Figures 2b and 2c). Lane 3 (a207) shows a 1.1-kb band and another band, 3.8-kb, that is slightly larger than expected. Lane 5 (f38) shows the 1.1-kb band and again a much larger band than expected, 5.3-kb. The unexpected bands in these two strains suggest an internal rearrangement of targeted *P*[*Ddc*] sequences such that larger *Eco*RI fragments were generated than predicted. If a complete *P*[inv*Ddc*] is present at *vg* from experiment 'x', a 3.5-kb band and a 5.0-kb band would be seen in lane 7 or 8 on the autoradiograph due to the *Eco*RI sites in *P*[inv*Ddc*] (Figure 2d). Lane 7 shows a 1.8-kb band and lane 8 shows the expected 3.5-kb and 5.0-kb bands. The vg^+ probe, therefore, reveals that all four lines, a207, f38, x347.2, and x347.11 contain insertions at *vg*.

In order to demonstrate that the insertions at *vg* contained *Ddc* sequences in these lines, the blot was stripped and re-probed sequentially with restriction fragments from *Ddc* (Figure 2c). Any genomic *Eco*RI restriction fragment that contains both *Ddc* and *vg* sequences will hybridize to both the vg^+ probe and the respective *Ddc* probe. This will result in a comigration of bands on autoradiographs taken from the blot after hybridization of these probes. The first *Ddc* probe used was the 5' 2.5-kb *Pst*I/*Eco*RI fragment (hereafter referred to as the 5' *Ddc* probe) shown in Figure 2c. The resulting autoradiograph is shown in Figure 5b. A 7-kb band is seen in all lanes from the endogenous *Ddc* locus. Lanes 2 and 3 also show a band slightly larger than 7-kb due to the donor *P*[*Ddc*] at 21B. Lanes 4 and 5 show a 5.4-kb band due to the donor *P*[*Ddc*] at 35C. In lane 3 (line a207) a 3.8-kb band is present which is the same size as that observed when the vg^+ probe was hybridized to the blot. Lane 5 (line f38) shows a band of 5.3-kb that comigrates with the band seen when using the vg^+ probe. Lane 6 (*P*[inv*Ddc*](10C); Ddc^{ts2} *cn* vg^{21}/*CyO*) contains a 4.5-kb band due to the *P*[inv*Ddc*] on the X chromosome of this control strain. The original Southern performed with line 347 also showed the 4.5-

kb band in the heterogeneous stock demonstrating that the donor was still present in this line also (data not shown). Lane 7 (line x347.2) showed only the 7-kb band from the *Ddc* locus. Lane 8 (line x347.11) shows a 3.5-kb band that was also seen when using the vg^+ probe. The results employing the 5' *Ddc* probe indicate the presence of 5' *Ddc* sequences at *vg* in lines a207, f38, and x347.11.

The blot was stripped again and re-probed (data not shown) with the 4.2-kb *Eco*RI/*Pst*I *Ddc* fragment (hereafter known as the 3' *Ddc* probe) shown in Figure 2c. The only co-migration observed was a 5.0-kb *Eco*RI band in lane 8 (line x347.11). This indicates the presence of 3' *Ddc* sequences at *vg* in line x347.11. The combination of *vg* and *Ddc* probes suggests that line x347.2 does not contain *Ddc* sequences at *vg* but that a small insertion remains, probably consisting of the *P* element portion of *P*[inv*Ddc*].

The restriction maps of *P*[*Ddc*], *P*[inv*Ddc*], and *vg* , and the Southern analysis above, led us to predict that only line x347.11 had a complete *Ddc* gene inserted at *vg*. Further Southern analysis of lines a207, f38, and x347.11 was carried out to examine the size of the targeted *Ddc*-containing *P* elements. Genomic DNA samples from these lines were digested with *Pst*I which liberates a 7.6-kb fragment from both the *Ddc* locus and any complete *P*[*Ddc*] or *P*[inv*Ddc*]. The Southern blot was hybridized sequentially with the *Ddc* probes mentioned above and the central 0.9-kb *Eco*RI *Ddc* fragment shown in Figure 2c. All three probes hybridized to a single 7.6-kb band in line x347.11 confirming the integrity of *P*[*Ddc*] in this line. Preliminary DDC assays conducted on the stock x347.11/*CyO* suggest that the targeted *Ddc* gene is functioning at a near wild type level (Sandra O'Keefe personal communication). Line a207 revealed the same 7.6-kb band and a 4.1-kb band when using the 5' *Ddc* probe, the 3' *Ddc* probe, and the central 0.9-kb *Eco*RI *Ddc* probe indicating a large internal deletion of the *P*[*Ddc*] in vg^{a207}. Line f38 showed the 7.6-kb band with all three *Ddc* probes but showed a smaller 3.9-kb band with only the 5' and 3' *Ddc* probes, not the central 0.9-kb *Eco*RI *Ddc* probe. This indicates an internal deletion of the *P*[*Ddc*] at vg^{f38} that includes the central 0.9-kb *Eco*RI fragment of

Ddc. These observations confirm that x347.11 is the only targeted line with a complete *Ddc* gene at *vg* and that lines a207 and f38 contain targeted *P*[*Ddc*] elements with internal deletions.

Mobility of donor *P* elements: Is a transposition intermediate or a genomic copy of the donor *P* element used in the targeted transposition? If transposition intermediates are used, then mobility of the donors should parallel the rate of targeted transposition. Mobility of the donors was estimated using a transposition assay. Single fly DNA amplification was performed on segregants of experiments 'a', 'f', and 'x' that did not inherit the original donor *P* element containing chromosome. These were Ddc^{ts2} *pr cn* vg^{79d5}/ *CyO* progeny of the second cross from experiments 'a', 'f', and male progeny from the second cross of experiment 'x' (Figures 1, 3, and 4a). Appropriate individuals were tested for the presence of *P*[*Ddc*] (experiments 'a' and 'f') using primers #3 and #5 (Figure 2c) or for the presence of *P*[inv*Ddc*] (experiment 'x') using primers #2 and #5 (Figure 2c). If a product is observed, then a transposition of the donor element to another chromosome must have occurred. The results of all mobility experiments are shown in Table 2. The ability of *P*[*Ddc*](21B) to move to another chromosome was ten to twenty fold less than that for *P*[inv*Ddc*](10C) or *P*[*Ddc*](35C) although clusters of transpositions can not be ruled out. These results suggest that a transposition intermediate of the donor *P* element is not used for targeted transposition because the large difference between the mobility of *P*[*Ddc*](21B) and the other two donors does not correlate with the similar rates of targeted transposition.

TABLE 2

Donor transpositions

Donor	Transpositions	Flies tested
P[*Ddc*](21B)	1[a]	153
P[*Ddc*](35C)	11[a]	72
P[inv*Ddc*](10C)	12[b]	95

[a] Using primers #3 and #5

[b] Using primers #2 and #5

DISCUSSION

In this Chapter, a number variables were examined for their effect on the frequency of targeted transposition. Experiments 'a', 'f', and 'x' demonstrated that the rate of targeted transposition was similar when using donor elements either in a *cis* configuration, or on a non-homologous chromosome, and when using proximal versus distal donor elements *in cis*. The average rate was approximately 3 events per 7 x 10^3 flies produced. Although premeiotic clusters cannot be ruled out, they usually give rise to many identicle events and the targeted alleles obtained in this Chapter were generally different with respect to internal deletions or orientation of insertion. The screening of all flies with increased *vg* phenotypes as well as a significant number of borderline mutants using DNA amplification ensured that no targeting events were overlooked. However, the results showed that this rigorous screening was unnecessary since all nine of the targeted events reported in Table 1 produced a strong mutant phenotype.

In experiment 'x', the targeting was carried out in a vg^{21}/CyO heterozygote. The 't' experiment, in contrast, was performed in a vg^{21} heterozygote that contained a non-inverted homologous second chromosome. Another difference between experiments 'x' and 't' was the use of a donor *in trans* on a non-homologous chromosome relative to the target versus a donor *in trans* on the homologous chromosome. One reason for the failure of the 't' experiment to yield a single targeted event could be the lack of a *CyO* chromosome. The structure of the homologous chromosome is known to affect *P* element reversion at w^{hd} (Engels *et al.* 1990) and may also be important for targeted gene replacement. Since the *CyO* chromosome is re-arranged by five inversions and the *vg* locus is included in one of these inversions (Lindsley and Zimm 1992), it is possible that pairing of homologous *vg* sequences is disrupted in vg^{21}/CyO heterozygotes and that this may enhance the rate of targeted transposition. Another explanation for the failure of the 't' experiment is the location of the donor element. The presence of the donor *in trans* on

the homologous chromosome may reduce or eliminate targeted transposition compared to donors on non-homologous chromosomes. Finally, a combination of both variables may have caused the lack of targeting in the 't' experiment. This question is resolved in the next Chapter.

The mechanism of targeted transposition is unknown (a model is presented in the GENERAL DISCUSSION). Replacement of the vg^{21} *P* element may depend on a transposition intermediate or a genomic copy of the donor element. If a transposition intermediate is used, the level of that intermediate, as measured by the rate of transposition to a different chromosome, should parallel the frequency of targeting. The mobility experiment, which was designed to measure the rate of transposition, demonstrated that *P*[*Ddc*](21B) is approximately one order of magnitude less mobile than either *P*[inv*Ddc*](10C) or *P*[*Ddc*](35C). Yet, the frequency of targeted transposition was similar between all three of these donors (Table 1). These results indicate that targeted transposition does not use a donor in the form of a transposition intermediate but rather a genomic copy of a donor element.

The targeted transpositions at the *yellow* locus were described as conversion events (Geyer *et al.* 1988). Several pieces of data support the concept of a replicative, conversion-like mechanism for targeted transposition. First, the sequence polymorphism located at nucleotide #33 in the 5' end of the donor *P* elements (Heslip *et al.* 1992) was present in all seven of the targeted *vg* alleles which contained the donor element in the same orientation as the original *P* element in vg^{21}. This indicates that targeted transposition resulted in a replacement of the 5' *P* element sequences of vg^{21} with 5' *P* element sequences from the donor. Secondly, complete donor elements were still present at their original locations in lines a207, f38 and x347. It seems likely that homology between the vg^{21} *P* element and the *P* element sequences of *P*[*Ddc*] or *P*[inv*Ddc*] is used to guide the replacement of the target with a donor transposon. The fact that the *P* elements in vg^{f49}, vg^{x336}, and $vg^{x347.11}$ are in the opposite orientation relative to the

original *vg*^{21} *P* element indicates that no more than the 31-bp of inverted terminal repeat sequence is required for the event.

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Chapter 3

Targeted Transposition at the *vestigial* Locus is Homologue Dependent

INTRODUCTION

The development of gene targeting techniques to circumvent the variable position effects on engineered constructs is now underway. Replacing sequences at the endogenous locus and recovering *P* element constructs at a single genomic position are now possible using *P* elements as tools (Gloor *et al.* 1991, 1994; Nassif *et al.* 1994; Staveley *et al.* 1994).

Targeted transposition is the replacement of one *P* element with another. At the *vestigial* (*vg*) and *yellow* (*y*) loci, these replacements often cause an increase in mutant phenotype (Geyer *et al.* 1988; Williams *et al.* 1988). In previous chapters, targeted transposition was used to recover a *Dopa decarboxylase* containing *P* element, the donor element *P*[*Ddc*], at an allele of the *vestigial* locus, the target vg^{21}. In most of these previous experiments, vg^{21} was heterozygous with a wild type *vg* allele on the multiply inverted second chromosome balancer (*CyO*). Under these conditions, the frequency of targeted transposition was approximately 3 per 2300 chromosomes scored when using donors in three different locations. However, a targeting experiment with a donor (*P*[*Ddc*](21B)) *in trans* to the target on a non-inverted homologous second chromosome was also conducted. This experiment yielded no targeted transpositions. It was impossible to determine whether the *trans* location of the donor on the homologue of the vg^{21} chromosome or the use of a non-inverted second chromosome heterozygous to the target or both variables was the reason for this result. In the experiments presented in this Chapter, this dilemma is resolved by targeting over a non-inverted chromosome using *P*[*Ddc*](21B) in a *cis* position relative to vg^{21}. Interestingly, targeted transposition occured frequently in this experiment indicating that the *trans* location of the donor *P*[*Ddc*](21B) on the homologue reduced this frequency. The data lead to the conclusion

that the frequency of targeted transposition is dependent on the nature of the homologous second chromosome.

The effect of using a hemizygous vg^{21} allele on targeted transposition was also addressed. An experiment consisting of vg^{21} heterozygous to a deficiency was conducted and the results indicated that targeted transposition occurs least frequently when the target is hemizygous.

These experiments demonstrated that targeted transposition is dependent on the homologous chromosome. This Chapter also includes a molecular characterization of targeted alleles and an evaluation of the ability of the vg^{21} *P* element to transpose when heterozygous to three different chromosomes. The mobility of the target vg^{21} *P* element was unaffected by the homologue, ruling out the possibility that altered transposition rates of the target *P* element may be the cause of the various targeted transposition frequencies.

MATERIALS AND METHODS

***Drosophila melanogastor* stocks :** All crosses were performed at 22° to 24° on yeast medium (Nash and Bell 1968) in batches unless otherwise noted. Ddc^{ts2} and Ddc^{n7} are temperature sensitive and recessive null alleles, respectively, of the *Ddc* gene (Wright *et al.* 1981). The vg^{21} allele is a *P* element insertion into the *vg* locus and vg^{79d5} is caused by a small deletion within the locus (Williams and Bell 1988). The third chromosomes, ry^{506} *P*[ry^{+} Δ2-3](99B) and *Sb* ry^{506} *P*[ry^{+} Δ2-3](99B), contain a stable genomic source of transposase (Robertson *et al.* 1988) and will be referred to as [Δ2-3] and *Sb*[Δ2-3] respectively. The donor, *P*[*Ddc*], is a *P* element vector, pHDlac, containing a 7.6-kb *Pst*I fragment that includes the *Ddc* gene (Chen and Hodgetts 1987). Other mutant alleles and chromosomes are described in (Lindsley and Zimm 1992).

DNA manipulations: Culturing and storage of bacteria, preparation of plasmid DNA, and restrictions were performed according to standard methodology (Maniatis *et al.*

1982). *D. melanogastor* genomic DNA for Southern hybridization was isolated as in Heslip *et al.* (1992).

Genomic DNA to be used for DNA amplification was isolated as follows: Flies were homogenized in 50 µl of 10 mM Tris-Cl pH 7.5, 60 mM EDTA, 0.15 mM spermidine, 0.15 mM spermine, and 100 µg/ml proteinase K. After incubating the homogenate for 30 to 60 min. at 37°, 50 µl of 0.2 M Tris-Cl pH 9.0, 30 mM EDTA, 2% SDS was added and incubated at 65° for 30 min. with occasional agitation. A single organic extraction was performed; 150 µl of 1:1 phenol/chloroform was added, mixed and warmed to 65° with occasional agitation. After brief centrifugation, 80 µl of the aqueous phase was subjected to DNA purification using GeneClean (Bio 101 Inc.) according to the manufacturer's instructions. The purified DNA was resuspended in 30 µl of TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Genomic DNA was extracted from single flies that were used to establish lines (see RESULTS). Flies with a borderline increase in mutant *vg* phenotype were pooled using 2-4 individuals in a single DNA extraction.

Southern hybridizations: Restricted DNA was fractionated by agarose gel electrophoresis and transferred onto Genescreen Plus membranes (Dupont). Hybridizations were performed at 42° in 50% formamide according to the manufacturer's instructions using oligolabeled DNA restriction fragments (Feinberg and Vogelstein 1983) purified from agarose gels with GeneClean (Bio 101 Inc.).

DNA sequencing: DNA amplification products were purified from agarose gels with GeneClean and sequenced using the ABI Taq DyeDeoxy Terminator Cycle Sequencing Kit according to the manufacturer's instructions. The sequencing reactions were fractionated and analysed on an ABI 370A DNA sequencing apparatus.

DNA amplification: Each amplification reaction used 3 µl (approximately 50 ng) of DNA in a total volume of 30 µl which contained 2-3 U of Taq polymerase (Bio-Can or BRL) and a final concentration of 50 mM Tris-Cl (pH 8.6), 1.5 mM $MgCl_2$, 0.005% ß-mercaptoethanol, 0.1 µg/µl bovine serum albumin (Boehringer Mannheim), 200 µM of

dATP, dCTP, dGTP, dTTP, and 0.3 ng/µl of primer. Reactions were run in a Stratagene Robocycler 40. The amplification program started with 5 min at 95°, 1.5 min at 60°, and 3 min at 73°, followed by 29 cycles of 1 min at 93°, 1 min at 60°, and 2-3 min at 73°. All amplification products were run on agarose minigels at 70 V. Primers used are listed by numbers as they appear in the results: #1; 5'-ATCCCGCGCGGCGGTGAGAG-3', #2; 5'-TTCGGAGCGTGATGTTGACA-3', #3; 5'-ATCGGCGTTGTAAAGACTGC-3', #4; 5'-GTACTCCCACTGGTATAGCC-3', #5; 5'-TAACATAAGGTGGTCCCGTC-3', #6; 5'-AATCAAGTGGGCGGTGCTTG-3'.

RESULTS

Targeted transposition using a non-inverted homologous chromosome: In previous experiments, the donor element, *P*[*Ddc*](21B), was located *in cis* or *in trans* relative to the target, vg^{21} (Experiments 'a' and 't', respectively of Chapter 2). Although three targeted alleles were obtained from experiment 'a', it could not be discerned which variable caused the lack of targeted transposition in experiment 't'. To address this issue, the following experiment was conducted. Crosses were set up (Figure 1a) such that a chromosome containing the donor, *P*[*Ddc*](21B), and the target, vg^{21}, was heterozygous to a non-inverted chromosome containing a vg^{+} allele in the presence of the transposase source, *Sb* Δ2-3 (see the males in cross ii of Figure 1a). Some of the progeny from these males could contain a targeted *vg* allele and are denoted as vg^{k*} (Figure 1a). Based on previous experiments, an increase in mutant *vg* phenotype, compared to the normally weak phenotype of the vg^{21}/vg^{79d5}, indicates a targeted event. Phenotypically strong *vg* males which did not inherit the *Sb* Δ2-3 chromosome were used to establish lines by mating them to Ddc^{ts2} *pr cn* vg^{79d5}/*CyO* females maintaining the desired chromosome over the *CyO* balancer chromosome. All progeny from cross ii (Figure 1a) with increased

Figure 1:

Crossing scheme for targeting *in cis* over the normal chromosome (a) or over the chromosome containing the vg^{B} deficiency (b). Males and females are indicated. The targeting crosses are designed to target *P*[*Ddc*], located at 21B, into vg^{21}. This occurs in the germline of the males in the second cross. The progeny from the second cross inheriting the putative targeted allele, vg^{k*} (a) or vg^{d*} (b), are detected by their more extreme mutant *vg* phenotype and used to establish stocks. The *CyO* second chromosome is marked with *cn* and *pr*. The *TM2* third chromosome is marked with *Ubx* and *e*.

a

♀ $P[Ddc](21B)\ Ddc^{ts2}\ pr\ vg^{21}$ X ♂ $\frac{Sp\ vg^{+}}{CyO}$; $\frac{Sb[\Delta 2\text{-}3]}{TM6,\ Ubx,\ e}$

♂ $\frac{P[Ddc](21B)\ Ddc^{ts2}\ pr\ vg^{21}}{Sp\ vg^{+}}$; $\frac{Sb[\Delta 2\text{-}3]}{+}$ X ♀ $\frac{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}{CyO}$

$\frac{P[Ddc](21B)\ Ddc^{ts2}\ pr\ vg^{k*}}{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}$; $\frac{Sb[\Delta 2\text{-}3]\ \text{or}\ +}{+}$

b

♀ $P[Ddc](21B)\ Ddc^{ts2}\ pr\ vg^{21}$ X ♂ $\frac{vg^{B}}{CyO}$; $\frac{Sb[\Delta 2\text{-}3]}{TM6,\ Ubx,\ e}$

♂ $\frac{P[Ddc](21B)\ Ddc^{ts2}\ pr\ vg^{21}}{vg^{B}}$; $\frac{Sb[\Delta 2\text{-}3]}{+}$ X ♀ $\frac{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}{CyO}$

$\frac{P[Ddc](21B)\ Ddc^{ts2}\ pr\ vg^{d*}}{Ddc^{ts2}\ pr\ cn\ vg^{79d5}}$; $\frac{Sb[\Delta 2\text{-}3]\ \text{or}\ +}{+}$

mutant *vg* phenotype were screened for targeted transposition using DNA amplification. The crosses shown in Figure 1a and subsequent molecular analyses constitute experiment 'k'.

Molecular maps of vg^{+} and vg^{21} are shown in Figures 2a and 2b. The cryptic vg^{21} allele is caused by the insertion of a 687 bp *P* element near the putative transcription start site (Williams et al. 1991). Figure 2c illustrates the molecular map of a targeted insertion at *vg* when the *P* element sequence of *P*[*Ddc*] resides in the same orientation as the original vg^{21} *P* element. The three primers used for single fly DNA amplification are also shown. These oligonucleotides are complementary to downstream *vg* (#1), 5' *Ddc* (#2), and 3' *Ddc* (#3) sequences, respectively. Following the amplification reaction, a product will be observed only in flies bearing a targeted transposition in which one end of the *Ddc* gene lies next to the downstream *vg* primer. Using both *Ddc* primers and the *vg* primer ensures that a product will be seen regardless of the orientation of the *P*[*Ddc*] in a targeted allele. It is important to note that a targeted insertion that does not consist of *Ddc* sequences next to the *vg* primer will not be detected using this molecular screen. A total of 2335 chromosomes were scored from experiment 'k' (Table 1). Genomic DNA from 94 flies with an increased mutant *vg* phenotype was subjected to amplification. The results are shown in Table 1. A total of 26 targeted alleles were obtained from experiment 'k'. Premeiotic clusters cannot be ruled out, however, the targeted alleles obtained have several differences among them including the presence or absence of one end of the *P*[*Ddc*], the presence or absence of the polymorphism in the *P* element portion of *P*[*Ddc*], and the different orientations of the insertions. Therefore, most of the targeted alleles are probably from independent events. The data suggest that the lack of targeted transposition in experiment 't' of our previous report was due to the *trans* location of the donor element relative to the target.

Figure 2:

Restriction maps of the *vestigial* alleles in this Chapter. Restriction sites are as follows: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; T, *Sst*I. All *Eco*RI sites are shown but only informative sites are shown for the rest, including the characteristic *Hin*dIII at the 5' end of *P* elements. (a) The vg^{+} allele. The vg^{+} probe referred to in the text is the 0.7 kb *Eco*RI fragment from this gene. The wavy arrow indicates the 5' end of the first exon of *vg* (Williams et al. 1991). (b) The vg^{21} allele. The 5' and 3' ends of the *P* element are indicated below the solid arrows (◄——►) on the map. The 3' end of the *P* element is about 20 bp upstream of the first exon of *vg* (Williams et al. 1991). (c) The 5' and 3' ends of the *P* element vector of *P*[*Ddc*] are shown. The wavy arrow indicates the transcription start site of *Ddc* and therefore the polarity of the *Ddc* gene is opposite to that of the *P* element. The putative *vg* transcription start site is not shown but remains unchanged from (b). Restriction map symbols: *vg* genomic DNA, ——; *P* element DNA with a terminus, ——►; *Ddc* exons and introns, —▬▬—. Below the restriction map are the locations and polarity (5' to 3') of the amplification primers whose sequences are listed in the MATERIALS AND METHODS. Below the primers are the locations of probes for Southern hybridizations.

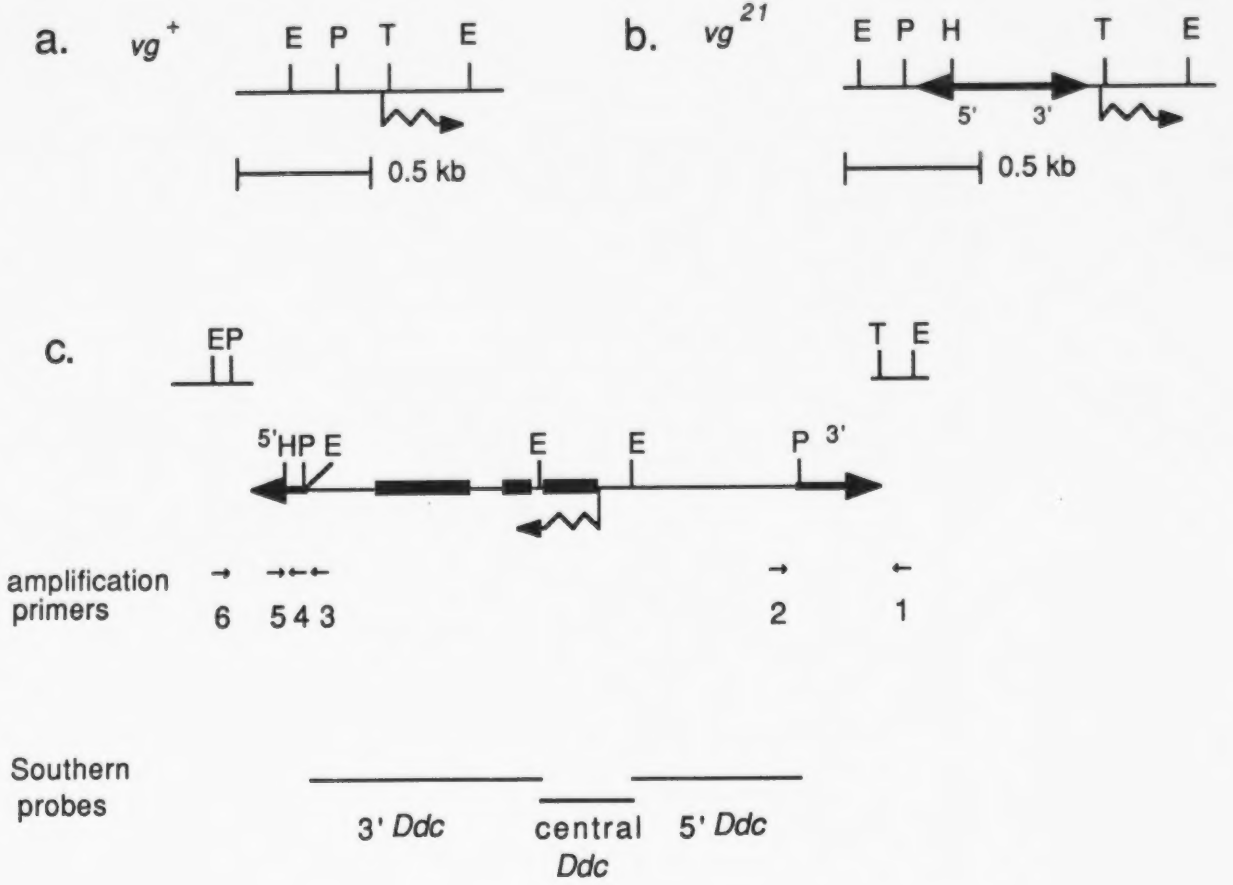
a.
vg+
E P T E
0.5 kb
b.
vg21
E P H T E
5' 3'
0.5 kb
c.
EP
T E
5'HP E
E
E
P 3'
amplification primers
6 5 4 3
2
1
Southern probes
3' Ddc
central Ddc
5' Ddc

Table 1
DNA Amplifications

Experiment	Allele	Amplification reaction primers[a]					Chromosomes scored	Nucleotide #33[b]
		#1, #2, and #3	#1 and #4	#4 and #6	#1 and #2	#1 and #3		
k	*k10*	+	+	-	-	+	2335	
	k19	+	-	-	+	-		
	ks2	+	-	+	+	-		A
	ks4	+	-	+	+	-		A
	ks6	+	-	+	+	-		A
	ks7	+	-	+	+	-		T
	ks8	+	-	+	+	-		A
	ks9	+	-	+	+	-		T
	ks11	+	-	+	+	-		T
	ks12	+	-	+	+	-		A
	ks13	+	+	-	-	+		
	ks14	+	+	-	-	+		
	ks15	+	-	-	+	-		
	ks16	+	-	+	+	-		A
	ks17	+	-	+	+	-		T
	ks18	+	-	+	+	-		T

	ks19	+	-	+	+	-		A
	ks20	+	-	-	+	-		
	ks21	+	-	-	+	-		
	ks22	+	-	-	+	-		
	ks23	+	-	+	+	-		A
	ks24	+	-	-	+	-		
	ks25	+	-	+	+	-		A
	ks26	+	-	-	+	-		
	ks27	+	-	-	+	-		
	ks28	+	-	-	+			
	Total	26		3	13	23	3	Frequency[c]=1%
d	ds1	+		-	+	+	-	2625	T
						Frequency[c]=0.04%		

[a] The reactions including primers #1, #2, and #3 were used in the initial screen for targeted alleles while the next two reactions using primers #1 and #4, and primers #4 and #6 were used to determine the orientation of donor in targeted alleles. In some alleles, neither of the previous primer pairs yielded a product, therefore, in separate reactions primers #1 and #2 or primers #3 and #1 were used to confirm the orientation of these alleles

[b] The *P* element vector of *P*[*Ddc*] contains a polmorphic T residue at nucleotide #33 (O'Hare and Rubin 1983). The presence of the polymorphism is indicated by a T and the presence of the vg^{21} or wild type nucleotide #33 is indicated by a A.

[c] The frequency of targeted transposition is the percentage of the number of targeted alleles detected over the total number of chromosomes scored.

Targeted transposition using a hemizygous vg^{21} allele: Experiments on the reversion of w^{hd} suggest that the optimal conditions for targeted gene replacement (Engels *et al.* 1990) include a target that is heterozygous to a deficiency (or is hemizygous). To test the effect of using a vg^{21} allele heterozygous to a deficiency on the frequency of targeted transposition, the crosses shown in Figure 1b were carried out. Briefly, the males shown in cross ii consist of the same chromosome used in experiment 'k' heterozygous to a non-inverted chromosome containing the vg^{B} deficiency and a Δ2-3 transposase source. The progeny from the second cross were scored for an increase in mutant *vg* phenotype and targeted alleles were confirmed using the same procedure as in experiment 'k'. The data are grouped as experiment 'd' in Table 1. Approximately 30 flies were analyzed for targeted transposition. One targeted allele was obtained from 2625 chromosomes scored. The results of experiments 'k' and 'd' demonstrate that targeted transposition is homologue dependent and is most frequent when the target vg^{21} is heterozygous to a structurally normal chromosome which contains a vg^{+} allele.

The molecular analysis of targeted alleles: The molecular screen used to detect targeted transposition, which included primers #1, #2, and #3 (Figure 2c), discriminates between the possible orientations of a targeted donor based on the size of the amplification product. If the *P* element vector of *P*[*Ddc*] is inserted at *vg* in the same orientation as the original vg^{21} *P* element, then primers #1 and #2 will yield a 0.8-kb product. If *P*[*Ddc*] inserts in the opposite orientation then primers #1 and #3 will yield a 0.7-kb product. Based on the size of the DNA amplification products in experiment 'k', 23 of the targeted alleles contained the donor transposon in the same orientation as the original vg^{21} *P* element and 3 targeted alleles contained inverted target donors. To confirm the orientation of targeted donor elements, primer #4 and either primer #1 (the downstream *vg* primer in Figure 2c) or primer #6 (the upstream *vg* primer shown in Figure 2c) were used in separate DNA amplifications of each targeted allele. Primer #4 is

complementary to *P* element sequences present in *P*[*Ddc*] but absent in the vg^{21} *P* element and directs synthesis toward the 5' *P* element terminus. In these reactions a product is expected in only one of the two reactions carried out on each line. A product from primers #4 and #6 indicates that the targeted *P* element is in the same orientation as the original vg^{21} *P* element whereas a product from primers #4 and #1 indicates the targeted *P* element is in the opposite orientation. The results, shown in Table 1, indicated that eleven alleles, *k19*, *ks15*, *ks20-ks22*, *ks24*, and *ks26-ks28*, yielded no product from either primer pair used to confirm orientation, presumably because sequences to which primer #4 anneals were deleted. These alleles were tested again using two separate reactions containing the downstream *vg* primer and either of the *Ddc* primers, #2 or #3, which anneal to internal donor sequences, to confirm their orientation. The orientation of the donor elements in 23 of the targeted alleles is the same as the original vg^{21} *P* element.

The DNA amplification products from reactions using primers #4 and #6 on 14 targeted alleles were purified from agarose gels and sequenced. A sequence polymorphism exists in *P* element vectors at nucleotide position 33 (O'Hare and Rubin 1983). The presence of this fortuitous polymorphism has implications for the mechanism of targeted transposition, and, if present in the targeted allele, demonstrates the replacement of the 5' end of the vg^{21} *P* element with the 5' end of *P*[*Ddc*]. The polymorphism specific to *P*[*Ddc*] was present in 5 of the 14 alleles sequenced (Table 1).

The single targeted allele from experiment 'd' contained *P*[*Ddc*] in the same orientation as the original vg^{21} *P* element and the polymorphism at nucleotide #33 of the transposon sequence was also present (Table 1).

The DNA amplification products of vg^{ks13} and vg^{ks14} using primers #4 and #1 were smaller than expected if the 5' end of *P*[*Ddc*] were to replace the 3' end of the original vg^{21} *P* element. Therefore, these products were also sequenced. The sequence indicated that the 5' *P* element portion *P*[*Ddc*] in these targeted alleles was missing and was replaced with DNA of unknown origin.

Southern analysis: Two established lines containing targeted *vg* alleles, vg^{k10} and vg^{k19}, were analyzed by Southern hybridization to determine the integrity of the targeted *Ddc* gene. Genomic DNA samples from these lines as well the wild type strain *Canton-S* and the parental stock from cross i of each targeting experiment, *P*[*Ddc*](21B) *pr* Ddc^{ts2} vg^{21}/*CyO*,were digested with *Pst*I which liberates a 7.6-kb fragment from both the *Ddc* locus and any complete *P*[*Ddc*]. The Southern blot was hybridized sequentially with the *Ddc* probes shown in Figure 2c. The resulting autoradiographs indicate that vg^{k10} and vg^{k19} contain internally deleted *Ddc* genes missing 1 kb and 3 kb respectively (Figure 3).

Mobility of the target *P* elements in the presence of the three different homologues: To test the influence of the homologous chromosome on the mobility of the vg^{21} *P* element, single fly DNA amplification was performed on segregants of experiments 'k', and 'd', as well as segregants from experiment 'a' (see Figure 3 of Chapter 2). These were Ddc^{ts2} *pr cn* vg^{79d5}/ *CyO* progeny of the second cross from each of the experiments which could not have inherited the original vg^{21}-containing chromosome. Appropriate individuals were tested for the presence of the 687 bp *P* element using primer #5 which is complementary to the last 20 nucleotides of the inverted terminal repeats. If a product is observed from these reactions then a transposition of the vg^{21} *P* element to another chromosome must have occurred. The results of all mobility experiments are shown in Table 2. The data indicate that the homologous chromosome does not influence the mobility of the vg^{21} *P* element .

Figure 3:

Southern hybridization of the established targeted lines. Genomic DNA samples were digested with *Pst*I. The blot was probed with the 5' *Ddc* probe shown in Figure 2c of Chapter 2. In order, the lanes contain DNA from *Canton-S*, *P*[*Ddc*](21B) vg^{21}/*CyO*, vg^{k10}/*CyO*, and vg^{k19}/*CyO*. The 7.6 kb band present in each lane is from the *Ddc* locus and any complete *P*[*Ddc*]. The smaller bands in the last two lanes indicate a deletion of *P*[*Ddc*] in these lines.

Canton-S
P[Ddc](21B) Ddc^ts2 pr vg^21
k10 vg^ /CyO
k19 vg^ /CyO
7.6 kb
6.5 kb
4.5 kb

Table 2

Target Mobility Assay

Experiment	a	d	k
Transpositions[a]	14	13	16
Tested[b]	37	36	36

[a] Transposition of the target *P* element from vg^{21} is detected by the presence of a 687 bp DNA amplification product using primer #5 (Figure 2c) Clusters of transpositions cannot be ruled out.

[b] The total number of flies tested for transposition of the target vg^{21} *P* element.

DISCUSSION

It has been particularly difficult to use *P* element transformation to study the regulation of expression of the *Ddc* gene. The promoter region of *Ddc* contains several motifs whose individual contribution to the regulation of the gene are similar in magnitude to the variation in expression due to position effects (Scholnick *et al.* 1986). In order to dissect these regulatory sequences, position effects must be eliminated or controlled.

Several variables effecting targeted transposition were studied in this Chapter in order to optimize this method as a means for controlling position effects. According to the results, the conditions yielding the highest frequency of targeted transposition include a donor element located *in cis* relative to the vg^{21} target and a non-inverted homologue (according to Lindsley and Zimm (1992) the *Sp* chromosome is cytologically normal) containing a vg^{+} allele heterozygous to that target. Targeted transposition occurs in approximately 1% of chromosomes scored (Table 1) under these conditions.

There is reason to believe that estimates of the frequency of targeted transposition herein are conservative. This is because the initial screen used to identify targeted alleles will produce a DNA amplification product only if the *Ddc* sequences in *P*[*Ddc*] that lie next to the specific *vg* primer used (primer #1, Figure 2c) are present. If a targeted allele contained a *P*[*Ddc*] donor element that was deleted for the end of the *Ddc* gene that was juxtaposed to the *vg* primer (primer #1) used then no DNA amplification product would be observed. The data presented in Table 1 demonstrate that nine of the targeted alleles containing the *P*[*Ddc*] in the same orientation as the original vg^{21} *P* element from experiment 'k' lacked the 5' *P* element sequences of *P*[*Ddc*]. This supports both the notion that some targeted alleles may contain only one end of *P*[*Ddc*] and that estimates of the frequency of targeted transposition presented here are conservative.

The frequency of targeted transposition in experiment 'k' (1%) was 25 times greater than that for experiment 'd' (0.04%, see Table 1). According to these frequencies, targeted transposition is homologue dependent.

In order to compare the results of the targeted transposition experiments in this Chapter to experiment 'a' (Chapter 2) which used the same donor (*P*[*Ddc*](21B)) *in cis* to vg^{21} but heterozygous to the multiply inverted balancer chromosome, *CyO* , a point must be made. In experiment 'a' (Chapter 2), 2980 chromosomes were scored. Three targeted alleles were obtained in that experiment giving a targeted transposition frequency of approximately 0.1%. This estimate of the targeted transposition frequency for experiment 'a' is a maximum, because a temperature shift was applied during the final cross which reduces the total number of chromosomes that could be scored. This makes the corrected estimate of the chromosomes scored a maximum and therefore the differences between experiments 'a' and 'k' conservative. Comparing the results of experiments 'k' and 'd', or, 'k' and 'a', targeted transposition is greater in experiment 'k' than either of the other two. Targeted transposition is most efficient when using a non-inverted homologue which contains a vg^{+} allele.

One explanation of the mechanism of targeted transposition is that the *vg* locus may behave like that of *singed* (*sn*). This locus has the peculiar property of attracting more that one *P* element such that two elements often reside next to each other (Hawley *et al.* 1988; Roiha *et al.* 1988). It is possible that *P*[*Ddc*] inserts next to the vg^{21} *P* element and that the original target element is then lost. However, there are two pieces of evidence which argue against this type of mechanism for targeted transposition at *vg*. First, if this mechanism operated then the status of the homologous second chromosome would not have any effect on the frequency of targeted transposition. Secondly, in the case of *sn*, several intermediates were observed in the process of one *P* element replacing the other, whereas in the case of *vg* no such intermediates have ever been observed.

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GENERAL DISCUSSION

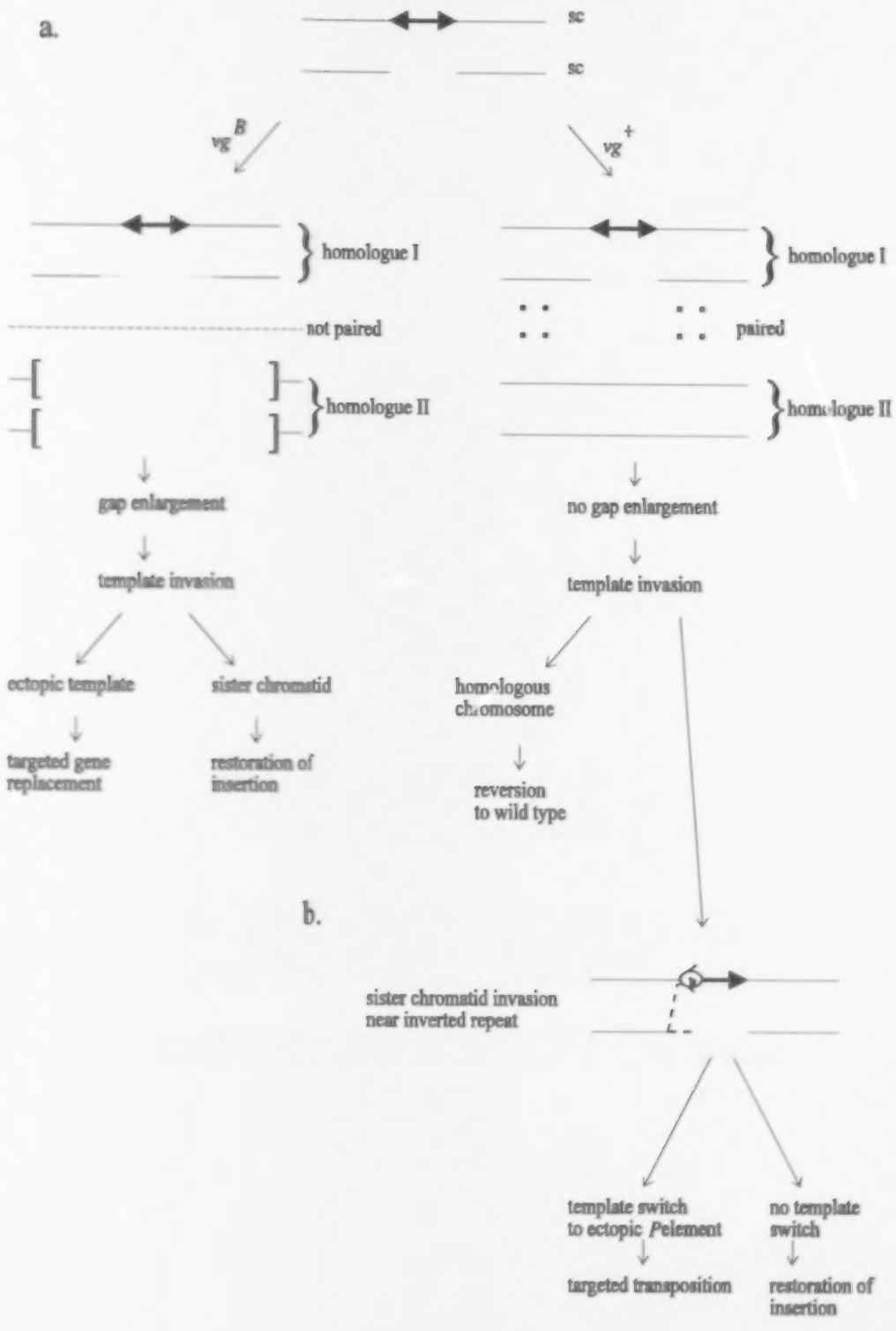
A General Model for Targeted Transposition

This thesis examined targeted transposition in *D. melanogaster*. Using data from these experiments a general model for the sequence of events surrounding excision and the possible decisions made concerning different pathways of repair is proposed in this final discussion. The majority of the data used to formulate this hypothetical view are from Chapter 3. Premeiotic clusters of events are expected to occur and would not be detected by the methods used because all crosses were performed in batches. The data presented therefore, may have large standard deviations. For the purpose of explaining targeted transposition, it is assumed that premeiotic clustering does not skew the results in Chapter 3 to an extent such that most of the targeted alleles obtained were clonal. The model explains data from a range of experiments on *P* elements and makes several testable predictions.

The first part of the model is a general explanation for the sequence of events that occurs after excision of a *P* element when heterozygous with either a deficiency or a wild type allele (Figure 1a). The first step in the process includes the assumption that targeted transposition occurs only after excision of the vg^{21} *P* element during the G2 phase of the cell cycle of pre-meiotic germ cells. Upon excision of the target *P* element (Figure 1a), the broken ends are used to search for homologous sequences to repair the lesion. Experiments on reversion at the *w* locus suggest that in most of these cases the sister chromatid, approximately 85%, or the homologue, approximately 15%, is used to repair the lesion (Engels *et al.* 1990). The next step in the model is a branch point that depends on the nature of the homologous chromosome. There is considerable evidence to suggest that *vg*, like other genes including *w* and *engrailed*, contains a strong pairing site (Hama *et al.* 1990; Williams *et al.* 1990; Gloor *et al.* 1991). Such sites are believed to facilitate tight pairing of homologues in their vicinity. In the first branch (the left branch of Figure 1a), the homologous chromosomes are not paired in the region of the excision because of the deficiency, vg^{B}. This lack of pairing induces a decision to start pruning back the broken ends using an exonuclease. This decision would be made before an attempt to use

Figure 1:

A general model for targeted transposition. The model does not address the actual molecular mechanisms involved but is meant to address the possible chain of events that occurs after the excision of a *P* element. (a) The first step is excision after DNA synthesis, only the vg^{21} homologue is shown (sc = sister chromatid). The branch point depends on the nature of the homologous second chromosome. The left branch can result in either targeted gene replacement or restoration of the original vg^{21} insertion. The right branch can result in reversion or (b). (b) There are two possible outcomes after the sister chromatid is invaded near the region of the terminal inverted repeat of the vg^{21} *P* element. The left branch results in targeted transposition and the right branch results in restoration of the original vg^{21} insertion.


a.
sc
sc
vg B
vg +
homologue I
homologue I
not paired
paired
homologue II
homologue II
gap enlargement
no gap enlargement
template invasion
template invasion
ectopic template
sister chromatid
homologous chromosome
targeted gene replacement
restoration of insertion
reversion to wild type
b.
sister chromatid invasion near inverted repeat
template switch to ectopic P element
no template switch
targeted transposition
restoration of insertion

the sister chromatid as a template for repair is initiated. New searches for homology would be started after the broken ends are degraded. Eventually, since no suitable template is found on the homologue, the sister chromatid or ectopic sequences may be used to repair the enlarged lesion. Using the sister chromatid would result in a restoration of the original *P* element (Engels *et al.* 1990) and is expected to be prevalent. Rarely, targeted gene replacement would result if ectopic sequences were used.

In the second branch of the model (the right branch of Figure 1a), the homologue is well paired, as in the case of a non-inverted chromosome containing a vg^+ allele. This pairing and the proximity of homologous sequences induces a decision not to delete sequences at the broken ends and either the homologue or the sister chromatid are used to supply a template for repairing the lesion (Engels *et al.* 1990). In the former case, the result is reversion of the insertion. This situation is analogous to the cases of high conversion of w^{hd} to altered w^{hd} alleles from the otherwise normal homologue where strong pairing exists (Johnson-Schlitz and Engels 1993). The evidence that the *w* locus contains a strong pairing site includes the fact that a *w* construct inserted into the *w* locus during transformation (Gloor *et al.* 1991).

In second part of the model (Figure 1b), the sister chromatid is used as a template after the decision to not prune the broken ends is made and explains both targeted transposition and internal deletion of *P* elements. In this case, a new strand displacement may occur a very small proportion of the time. The broken end invades the sister chromatid and after some polymerization, the newly sythesized strand invades the ectopic *P* element sequences of *P*[*Ddc*]. A precedent for strand displacement *in vitro* exists in bacteriophage T4 (Formosa and Alberts 1986) and has been used to explain targeted gene replacement and the formation of duplications within those experiments (Nassif *et al.* 1994). After further polymerization using *P*[*Ddc*] as a template and resolution of the replication bubble, the result is targeted transposition. The random occurrence of the donor specific polymorphism at nucleotide #33 in targeted *P* elements and the fact that

some targeted donor elements are inserted at *vg* in the opposite orientation relative to the vg^{21} *P* element suggest that a new strand invasion of the ectopic *P* element sequence occurs very early in the repair process. There is also the possibility that some heterduplex is formed at nucleotide #33 if the strand invading *P*[*Ddc*] has been extended up to that point. If both broken ends at the excision site invade homologous sequences on the sister chromatid and then both ends switch to the ectopic template but fail to use the entire *P*[*Ddc*] then an internally deleted donor will be found at *vg*. If only one end of the invaded sister chromatid re-invades *P*[*Ddc*] and fails to use the entire *P*[*Ddc*] as a template then only one side of *P*[*Ddc*] will be found at *vg*. In even fewer cases both ends may re-invade resulting in the use of the entire donor element as a template, resulting in a complete *P*[*Ddc*] at *vg*. If no re-invasion occurs, the entire *P* element residing on the sister chromatid or part thereof can be used to repair the lesion, resulting in restoration of the *P* element or an internally deleted *P* element in a manner already proposed by Engels *et al.* (1990).

There are several assumptions important to the model. First, targeted transposition occurs after excision of the target *P* element in G2 of the cell cycle. Secondly, after using the sister chromatid as a template for extended lengths, such as when the broken ends are already degraded (Figure 1a), a re-invasion of ectopic *P* element sequences does not occur. The model does not explain what happens to the chromatid in which the original excision took place.

The model is testable and predicts that targeted gene replacement and not targeted transposition will be favored at loci where pairing sites do not exist. In this case the left branch of the model in Figure 1a would be used more often. Furthermore, the model predicts that targeted transposition would not be homologue dependent at these loci. In addition, if a transposase source under the control of a cell cycle dependent promoter was used then the model predicts that targeted transposition would occur less frequently or never if the transposase source was expressed only in G1 of the cell cycle. Finally, using

a donor template containing a series of polymorphisms in the *P* element DNA (similar to the polymorphisms in the ectopic copy of *w* used by Gloor *et al.* 1990) would result in altered sequences copied into *vg* at a frequency that depends on their position in the sequence.

In support of the model, it is worthy to note that targeted transposition conducted over the multiply inverted chromosome, *CyO* (experiment 'a' of Chapter 2), which presumably is less able to pair in the *vg* region, resulted in a frequency between that of experiments 'k' and 'd' (Chapter 3). In experiment 'k', the target was heterozygous to a chromosome which should be well paired in the *vg* region. In experiment 'd', the target was heterozygous to a chromosome which would be completely unpaired at *vg* due to a deficiency in this region.

Two experiments used donors in a *trans* position relative to the target, 'x' and 't'. The results of these experiments are difficult to reconcile when considering that the frequency of targeted transposition was similar between experiments 'a', 'f', and 'x' yet the frequency of targeted transposition in 't' was much less. In targeted gene replacement experiments (Gloor *et al.* 1991), several donors gave higher rates of conversion than others nearby suggesting that some insertions may be hotspots for contributing information to repair an excision site. The X-linked donor in experiment 'x' may be such a hotspot. Further experiments using donors *in trans* will be needed to determine whether such hotspots exist or whether the reduced frequency of targeted transposition for experiment 't' is due to the fact that the donor is located on the homologous chromosome as opposed to a non-homologous chromosome. It is interesting to note that the conditions for optimal targeted transposition frequencies, namely the use of normal homologous chromosome, appear to be opposite those for targeted gene replacement (Engels *et al.* 1990; Gloor *et al.* 1991).

This thesis provides a basis for utilizing a new technique, namely targeted transposition, to control position effects. The experiments in Chapter 3 demonstrate that

targeted transposition can be efficient and convenient to use. Stocks were established for six targeted alleles (*28w, a207, f38, x347.11, k10,* and *k19*) and therefore the integrity of the targeted *Ddc* gene could be determined by Southern analysis for these lines. Two of these (*28w* and *x347.11*) contain complete *Ddc* genes at *vg*. Although the ability of other genes to be expressed at *vg* is unknown, preliminary experiments measuring the enzyme activity levels of DDC in vg^{28w} (Chapter 1) and $vg^{x347.11}$ (Chapter 2) indicate that *Ddc* is expressed normally when inserted at *vg* (Sandra O'Keefe and Don Price, personal communication). This indicates that targeted transposition is effective for studying the regulation of expression of *Ddc* and that different constructs targeted into *vg*, where position effects are minimal and constant, can be used to directly ascribe changes in regulatory sequences to changes in expression of this gene.

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